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2. Patent application number *(The Patent Office will fill in this part)* — **9914616.9**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UNIVERSITY OF SHEFFIELD
WESTERN BANK
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S10 2TN

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — ATTACHMENT SURFACE

5. Name of your agent *(if you have one)* — MARKGRAAF PATENTS LIMITED

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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ATTACHMENT SURFACE

The invention herein described relates to a surface to which cells, preferably mammalian cells, attach and proliferate and which enables said attached cells to detach therefrom such that said surface can be used in various therapeutic and cosmetic tissue engineering/surgical procedures.

Tissue engineering is an emerging science which has implications with respect to many areas of clinical and cosmetic surgery. More particularly, tissue engineering relates to the replacement and/or restoration and/or repair of damaged and/or diseased tissues to return the tissue and/or organ to a functional state. For example, and not by way of limitation, tissue engineering is useful in the provision of skin grafts to repair wounds occuring as a consequence of: contusions, or burns, or failure of tissue to heal due to venous or diabetic ulcers. Further, tissue engineering is also practised during: replacement of joints through degenerative diseases such as arthritis; replacement of coronary arteries due to damage as a consequence of various environmental causes (eg smoking, diet) and/or congenital heart disease including replacement of arterial/heart valves; organ transplantation; repair of gastric ulcers; replacement bone tissue resulting from diseases such as oesteoporosis; replacement muscle and nerves as a consequence of neuromuscular disease or damage through injury.

Unfortunately, the culturing of cells/tissues *in vitro* represents only part of the problem faced by tissue engineers. In many examples the growth of cells in culture is not the major obstacle. It is the transfer of the cells/tissue, via a suitable vehicle (for example and not by way of limitation culture wear,

prostheses, implants, 3- dimensional matrix supports, extracellular matrix protein coated dressing, bandages, plasters), so that the cells/tissue are incorporated into the patient to be treated which represents a further, more taxing problem. Vehicles suitable for the transfer of replacement tissue have to satisfy certain requirements if they are to be useful in tissue engineering. For example, transfer vehicles optionally, have the following characteristics;

i) they provide a surface to which cells may become securely attached;

ii) they allow attached cells to grow and divide unhindered by the attachment surface;

iii) where appropriate, they provide an attachment surface which does not influence the differentiated (or undifferentiated) state of the attached cells;

iv) they maintain cells in a sterile and immunologically silent status;

v) they are minimally toxic to the patient;

vi) they do not transmit bacterial or viral disease; and

vii) they provide a surface from which attached cells may easily detach and subsequently invade the tissue site requiring replacement, restoration or repair.

A number of surfaces have been identified which provide substrates on which cells may attach, grow and proliferate in culture and an excellent example of a cell type expressing the aforementioned characteristics is a keratinocyte.

The favoured substratum for supporting the attachment and proliferation and growth of is collagen I, but others have been investigated ( ). For example,

keratinocytes seeded or deposited onto collagen-glycosaminoglycan (C-GAG) substrates and grafted to burns to form a cultured skin substitute (CSS), developed into permanent skin tissue after 14-28 days ( ). Keratinocytes are also able to grow *in vitro* on synthetic hydrophilic polymer supports ( ). Keratinocytes have been grafted onto poly(hydroxyethyl methacrylate) supports and these have shown improved wound bed healing, with no difference in the cytokeratin pattern of the unreconstructed epidermis and normal human skin ( ). Previous work has shown how carboxylic acid-containing plasma co-polymers encouraged the attachment and proliferation of keratinocytes over a period of 7 days ( ).

The influence of extracellular matrix proteins on human keratinocyte attachment, proliferation and transfer to a dermal wound bed model has also been studied ( ). Matrigel, collagen I and IV were found to enhance initial attachment; RGD, vitronectin, fibronectin and irradiated 3T3 fibroblasts did not. Proliferation of cells was also found to be positively influenced (although to a lesser extent than initial attachment) on matrigel, collagen I and IV and irradiated 3T3 fibroblasts. Keratinocytes proliferating on the latter substrates maintained the ability to transfer to a simple *in vitro* wound bed model.

The culture of cells on defined-surfaces has rarely addressed the challenges of differentiated cells. Fibroblasts, for example, commonly used in studies on differentiated cell types, maintain considerable pleiotropy in culture and are relatively easy to culture. Keratinocytes in contrast will normally undergo programmed differentiation *in vivo* moving upwards from the basal epidermal layer (where they are in contact with the basement membrane

attached to the underlying dermis) to the upper epidermal layers. In the latter layers they lose their nuclei and undergo terminal differentiation. Once past a certain point of differentiation they are not capable of migration or proliferation and largely serve a barrier function for the skin.

Keratinocytes in culture undergo terminal differentiation in most culture conditions and will react to adverse conditions by premature differentiation. The challenge therefore is to establish a surface which, ideally, does not itself promote differentiation and which can, ideally in conjunction with appropriate culture media, maintain cells, such as keratinocytes, in a proliferative phenotype in which they are capable of attaching and then subsequently transferring to an *in vitro* wound bed model.

When considering tissue engineering and wound repair, several different approaches are available. Products and potential therapies currently being investigated generally fall into three categories: epidermal replacements, dermal replacements and skin substitutes.

Epidermal replacements consist of keratinocytes cultured as a sheet alone, or with a vehicle, and usually involve culturing autologous (patient's own) epithelial cells grown to confluence *in vitro*. Although non-autologous versions are available as "off the shelf" solutions, there is no evidence that non-autologous cells will take, although they can act as a biological bandage. For these reasons, non-autologous, products (Epicel™ and Acticel™) have received mixed clinical success. Another product under investigation, Laserskin, uses hyaluronic acid as a keratinocyte delivery system, but when used by those skilled in the art, the carrier is primed with a layer of irradiated

3T3 fibroblasts to promote keratinocyte proliferation. Others are also investigating film carriers for keratinocyte transfer prior to them forming an intact sheet.

Dermal replacements comprise a support structure, or implanted matrix, for infiltration, adherence, proliferation and neo-matrix production by fibroblasts (and in some cases endothelial cells). Integra™ uses a dermal component of bovine dermal collagen I crosslinked with chrondroitin-6-sulphate on a silicone backing sheet. Also under consideration is a seeded variant with fibroblasts and epidermal cells. The synthetic matrix degrades after 3/4 weeks and promotes neo-dermis formation prior to split-thickness mesh grafting. Alloderm™ is freeze-dried, human de-epidermised dermis containing donor fibroblasts (from screened skin bank donors). Xenoderm™ is similar, utilising a porcine dermis, which allows incorporation of the matrix into the wound bed, exhibits low immunogenicity and allows re-population with host cells. Others are also developing collagen based polymers as supports, or synthetic matrices, for the delivery of keratinocytes and fibroblasts which have been shown to support cell ingrowth when implanted unseeded into experimental wounds.

Currently considered the most promising product, Dermagraft™ utilises a PGA/PLA matrix seeded with allogenic fibroblasts. Complete resorption of the implanted matrix, after 4 weeks is seen, and cells deposit collagen I-III-VI, elastin, fibronectin and decorin. An unseeded version didn't support a graft take. The advantage of this product is that it can retard wound contraction if seeded with keratinocytes, and the non collageneous matrix overcomes problems associated with immunogenicity/BSE transfer.

Skin substitutes combine both the dermal and epidermal replacements. Appligraf™ combines collagen I gel seeded with allogenic fibroblasts with a confluent sheet of allogenic keratinocytes. Although there are questions about the long term survival of allogenic keratinocytes and fibroblasts in dermal lesions, it is possible that viable allogenic cells may deliver biological mediators (e.g. growth factors) capable of accelerating the repair process.

It will be apparent to one skilled in the art that the above solutions to providing wound healing systems (other than those which use material derived from the patients tissue, ie autologous tissue) suffers from the potential to transfer infective agents from the donating source to the patient to be treated. Additionally, xenografts still require general acceptance by the general public as an alternative to the use of human tissue in tissue engineering.

Several issues concerning wound pre-treatment, choice of matrix support (for cell growth) and the use of allogenic cells remain to be fully resolved, but there is little doubt that tissue engineered approaches to wound repair will present significant therapeutic benefits compared with existing treatments. It will be apparent from the above description, that keratinocytes provide an excellent model system for the study of tissue for use in tissue engineering. However an overriding problem faced by tissue engineers is the provision of a substrate which can satisfy all the requirements of the ideal vehicle for the culture and transfer of cells/tissues to a patient.

Cell culture wear and biological implants or vehicles are typically manufactured from or coated with polymers which allow the attachment, growth and proliferation of cells. Often, if the substrate/vehicle is to be used as means to implant the cultured cells/tissues, then the implanted matrix used in conjunction therewith, may be biodegradable (please see WO/9012603). Furthermore the treatment of substrates to encourage the attachment and proliferation of cells is well known in the art. For example WO89/02457 and WO90/02145 disclose the chemical modification of surfaces that facilitate the attachment of cells. WO89/02457 relates to the chemical modification of polytetrafluoroethylene (Teflon^{tm}) and other fluorocarbon polymers and its use in the culturing of endothelial cells. WO90/02145 describes the use of a co-polymer of neutralised perfluoro-3,6-dioxa-4-methyl-7-octene sulphonyl fluoride and the monomer, tetrafluoroethylene, for use in coating various types of substrate for use in cell/tissue culture.

US 4 919 659 describes the use of plasma polymerizable gases (eg acetone, methanol,ethylene oxide) to coat surfaces to which cells attach and grow. The coated surfaces show enhanced binding of fibronectin (a cell adhesion polypeptide) and hence facilitates the attachment of cells to the treated surfaces. The surfaces thus treated are useful in providing articles for biological implants and cell culturewear. Typically, materials such as polyester, tetrafluoroethylene or polyurethane are coated with a plasma polymerized gas and then contacted with fibronectin. The fibronectin adsorbed surfaces show enhanced attachment of mouse 3T3 cells when compared to control surfaces.

Plasmas are ionised gases, commonly excited by means of an electric field. They are highly reactive chemical environments comprising ions, electrons, neutrals (radicals, metastables, ground and excited state species) and electromagnetic radiation. At reduced pressure a regime may be achieved where the temperature of the electrons differs substantially from that of the ions and neutrals. Such plasmas are referred to as "cold" or "non-equilibrium" plasmas. In such an environment many volatile organic compounds (neat or with other gases, eg Ar) have been shown to polymerise (H. K. Yasuda, Plasma Polymerisation, Academic Press, London, 1985) coating both surfaces in contact with the plasma and those downstream of the discharge. The organic compound is often referred to as the "monomer". The deposit is often referred to as a "plasma polymer". The advantages of such a mode of polymerisation include: ultra-thin pinhole free film deposition; plasma polymers can be deposited onto a wide range of substrates; the process is solvent free and the plasma polymer is free of contamination.

We have exploited plasma polymer deposition to coat suitable substrates for use in cell/tissue culture ( ). Thin polymeric films can be obtained from the plasmas of volatile organic compounds (at reduced pressure of 10^{-2} mmbar and ideally, < 100^{0} C). In plasma polymer deposition, there is generally extensive fragmentation of the starting compound or ionised gas and a wide range of the resultant fragments or functional groups are undesirably incorporated into the deposit. We have shown that by employing a low plasma input power (low plasma power)/ monomer flow rate ratio it is possible to fabricate films with a high degree of functional group retention. An example of such a low power/rate ratio is 2W and a flow rate of 2.0sccm.

However, other relatively low ratios may be used and are known to those skilled in the art.

This has been demonstrated for acrylic acid ( ). Co-polymerisation of acrylic acid with a hydrocarbon (e.g. 1,7-octadiene) allows a degree of control over surface functional group concentrations in the resultant plasma copolymer (PCP) ( ). PCPs can be deposited directly onto most surfaces, regardless of geometry, making them ideal for treating surfaces such as gauzes and fibres, as well as plasticware for cell culture. This would obviously make them useful for clinical applications where cells could be grown on PCP-coated –2 dimensional or 3-dimensional supports prior to application to wound beds or sites of tissue repair/restoration.

We have cultured human keratinocytes on surfaces which have been coated with plasma polymer/co-polymer. The use of a low power/monomer flow rate ratio produces a plasma polymer/co-polymer in which the acid functionality of the acid-containing monomer (in this example, acrylic acid) is largely preserved intact (retained) from the plasma-gas to the plasma polymer/co-polymer deposit. These deposits do contain other functional groups (e.g. hydroxyls arising from post plasma oxidation) but are described as "high acid functionality", reflecting the high degree of acid retention from the plasma gas into the plasma polymer film. "High acid functionality" does not refer to the amount (concentration/density) of acid functionality in the plasma polymer/co-polymer, which depends upon the co-polymerisation ratio of the acid-containing monomer/hydrocarbon.

Keratinocytes cultured on these surfaces not only attach, grow and proliferate in an undifferentiated state but also detach from the surface and transfer to a wound bed model. Surfaces that promote keratinocyte transfer in this manner show great promise in the field of wound healing. We ascribe these favourable characteristics to the high acid functionality of our treated surfaces and to the nature of the attachment surfaces in facilitating detachment of cells

Reference herein to high acid functionality is intended to include surfaces which have amounts of 5-20% surface acid functionality and more ideally in excess of 20% surface acid functionality. The percentages refer to the percent of carbon atoms in this type of environment. For example, 20 % acid functionality means that 20 of every one hundred carbons in the plasma polymer is in an acid-type environment.

It is an object of the invention to provide a surface to which cells may attach and proliferate.

It is yet still a further objection of the invention to provide a surface from which said attached cells may detach.

According to a first aspect of the invention there is provided at least one cell culture surface to which at least one cell can releasibly attach, characterised wherein said surface provides a high acid functionality.

Reference herein to high acid functionality relates to surfaces which contain between 5-20% surface acid functionality and more ideally still greater than 20% surface acid functionality.

It will be apparent to one skilled in the art that an acid functionality at attachment surfaces results in enhanced attachment of cells (Daw *et al* Biomaterials (1998), 19, 1717-1725). We have found that plasma polymerisation of cell culture surfaces at low plasma power/monomer flow rate ratio results in a retention of high acid functionality at surfaces coated with the polymer. Cells cultured on cell culture surfaces treated with 100% acrylic acid exhibit enhanced detachment from the treated surface thereby promoting keratinocyte infiltration of de-epidermised dermis (DED) (although the plasma polymer produced from 100% acrylic acid may not contain the optimal percentage of acid functionality for cell attachment). However, plasma co-polymerisation of acrylic acid with hydrocarbon, for example and not by way of limitation, 1,7-octadiene, allows a degree of control over the deposition process and the provision of a surface to which keratinocytes may attach, proliferate and detach therefrom. Typically, cell culture surfaces which have been treated with an excess of 50% acrylic acid in the monomer flow produce plasma polymer surfaces with between 5-21% acid functionality, depending on the concentration of acid used. For example, a surface treated with 100 % acrylic acid produces an acid surface functionality of approximately 21%.

In a preferred embodiment of the invention said surface provides a substrate onto which at least one cell can grow and proliferate. Preferably said surface facilitates growth and proliferation of said cell in an undifferentiated state.

Alternatively, said surface facilitates the growth and proliferation of said cells in a differentiated state, depending on tissue type.

In yet a further preferred embodiment of the invention said surface does not elicit an immune reaction in the cells attached thereto so that these do not provoke an immune reaction when they are delivered to a patient.

In yet still a further preferred embodiment of the invention said surface has minimal patient toxicity and so does not elicit an unfavourable reaction when cells attached thereto are delivered to a patient.

In yet a further preferred embodiment of the invention said surface is suitable for use with cells of mammalian origin, and more preferably cells of human origin.

In a further preferred embodiment of the invention said surface is suitable for use with any one of the following cell types; keratinocytes; chondrocytes; osteoblasts; endothelial cells. Ideally said cell is a keratinocyte.

In yet a further preferred embodiment of the invention said surface acid functionality is provided by a carboxylic acid functionality

In yet still a further preferred embodiment of the invention said surface acid functionality is between 5-20% surface acid functionality. More ideally still said surface acid functionality is greater than 20%. Ideally said acid functionality is provided by acrylic acid.

In yet still a further preferred embodiment of the invention , typically said surface is provided by coating a substrate with a plasma co-polymer of an acid containing monomer. For example and not by way of limitation, acrylic acid and a hydrocarbon, for example and not by way of limitation, 1,7-octadiene. Ideally said acrylic acid is provided at 50-100% and 1,7-octadiene is provided at 0-50% in the gas feed.

It will be apparent to one skilled in the art, that the cell culture surfaces of the invention are useful in clinical applications where cells could be grown on coated substrates prior to application to, for example and not by way of limitation, acute and/or chronic and/or minor and/or severe cutaneous wounds (including venous and diabetic ulcers); and/or cartilage repair; and/or bone repair; and/or muscle repair; and/or nerve repair; and/or connective tissue repair; and/or blood vessel repair. The invention also provides any of the aforementioned cell culture surfaces by providing said surfaces as an integral part of a tissue engineering vehicle.

According to a second aspect of the invention there is provided a vehicle for use in tissue engineering wherein said vehicle has integral therewith, or applied thereto, a cell culture surface to which at least one cell can reversibly attach characterised in that said surface has a high acid functionality.

Vehicle may be defined as any means by which cells cultured on a surface according to the invention may be used in tissue engineering. For example and not by way of limitation, a prosthesis, implant, matrix, stent, culture dishes, gauze, bandage, plaster, biodegradable matrix.

In a preferred embodiment of the invention there is provided a therapeutic vehicle comprising a surface according to the invention to which is attached selected cell(s) wherein said therapeutic vehicle is adapted to be applied and/or implanted into a patient requiring therapeutic tissue engineering.

In yet a further preferred embodiment of the invention there is provided a therapeutic vehicle comprising a suitable matrix material (for example, and not by way of limitation a matrix material which is synthetic or naturally occuring and either long-lasting or biodegradable) having incorporated and/or embedded therein and/or associated therewith and/or attached thereto a surface according to the invention to which is attached cells for use in surgical implantation procedures.

In yet still a further preferred embodiment of the invention said vehicle is suitable for use with any one of are the following cell types; keratinocyte, chondrocyte, osteoblast, endothelial cell.

In yet a further preferred embodiment of the invention said therapeutic vehicle comprises keratinocytes incorporated and/or embedded and/or associated and/or attached to a surface according to the invention for use in the treatment of wounds. More preferably still said therapeutic vehicle is adapted for the treatment of burns.

According to a third aspect of the invention there is provided a cosmetic vehicle comprising a cell culture surface according to any aspect or embodiment of the invention for use in cosmetic tissue engineering.

According to a fourth aspect of the invention there is provided a method of preparing a surface according to any previous aspect or embodiment of the invention comprising;

i) mixing a selected ratio of acid containing monomer and a hydrocarbon in a gas feed;

ii) creating a plasma of said mixture; and

iii) coating a suitable substrate with said plasma to provide a surface polymer/copolymer retaining high acid functionality.

Preferably said plasma power is created using a plasma power of 0 –50W and a flow rate of 0 – 20 sccm, under continous wave conditions. However, in the instance of where a pulse wave is used corresponding corrections are made to the plasma power and flow rate as is known by those skilled in the art.

In a preferred method of the invention said acid is acrylic acid and said hydrocarbon is 1,7-octadiene.

In a further preferred method of the invention said plasma comprises 50-100% acrylic acid and 0-50% 1,7-octadiene in the gas feed.

In yet a further preferred embodiment of the invention said plasma comprises the following ratios of acrylic acid and 1,7-octadiene;

Acrylic acid %	1,7-octadiene %
50	50

60	40
70	30
80	20
90	10
100	0

in the gas feed.

An embodiment of the invention will now described, by example only and with reference to the following tables and figures;

Table 1 shows X-ray photoelectron spectroscopy of PCP's formed from acrylic acid and 1,7 octadiene (Faa/Ftot = molar fraction of acrylic acid in the plasma gas feed);

Table 2 shows the adherence of keratinocytes to DED after transfer from various attachment surfaces; % values correspond to the amount of acrylic acid in the plasma feed gas;

Figure 1 shows the attachment of keratinocytes to various surfaces; % values correspond to the amount of acrylic acid in the plasma feed gas;

Figure 2 is a measure of keratinocytes remaining attached to a surface after transfer to DED; % values correspond to the amount of acrylic acid in the plasma feed gas;and

Figure 3 is a measure of keratinocyte retention on DED after transfer; % values correspond to the amount of acrylic acid in the plasma feed gas

Materials and Methods

Plasma Co-Polymerisation

Acrylic acid and 1,7-octadiene were obtained from Aldrich Chemical Co. (UK). All monomers were used as received, after several freeze-pump/thaw cycles. Polymerisation was carried out in a cylindrical reactor vessel (of 8 cm diameter and 50 cm in length), evacuated by a two stage rotary pump. The plasma was sustained by a radio-frequency (13.56 MHz) signal generator and amplifier inductively coupled to the reactor vessel. The base pressure in the reactor was 3×10^{-3} mbar.

Acrylic acid and 1,7-octadiene were co-polymerised at a plasma power of 2 W and a total flow rate of 2.0 sccm. Plasma co-polymers were deposited onto a carrier polymer, polyhydroxybutyrate (Goodfellow, Cambridge, UK) and clean aluminium foil (for XPS analysis). The pressure during co-polymerisation was typically 4.0×10^{-2} mbar.

For all co-polymerisations, a deposition time of 20 minutes was used. The monomer mixtures were allowed to flow for a further 20 minutes after the plasma was switched off. This was done in an attempt to minimise the up-take of atmospheric oxygen by the deposits on exposure to the laboratory atmosphere.

X-ray Photoelectron Spectroscopy

XP spectra were obtained on a VG CLAM 2 photoelectron spectrometer employing Mg K α X-rays. Survey scan spectra (0-1100 eV) and narrow spectra were acquired for each sample using analyser pass energies of 50 and 20 eV respectively. Spectra were acquired using Spectra 6.0 software (R. Unwin Software, Cheshire, UK). Subsequent processing was carried out with Scienta data processing software (Scienta Instruments, Uppsala, Sweden). The spectrometer was calibrated using the Au 4f 7/2 peak position at 84.00 eV, and the separation between the C 1s and F 1s peak positions in a sample of PTFE measured at 397.2 eV, which compares well with the value of 397.19 eV reported by Beamson and Briggs ( ).

Cell Culture

Normal human adult keratinocytes (obtained from breast reductions and abdominoplasties) were isolated from the dermal/epidermal junction as previously described.[14] Cells were cultured in complete Green's media, which included cholera toxin (0.1 nM), hydrocortisone (0.4 μg/ml), EGF (10 ng/ml), adenine (1.8×10^{-4} M), triiodo-l-thyronine (2×10^{-7} M), insulin (5 mg/ml), transferrin (5 μg/ml), glutamine (2×10^{-3} M), fungizone (0.625 μg/ml), penicillin (1000 IU/ml), streptomycin (1000 μg/ml) and 10 per cent fetal calf serum. Cells were cultured at 37°C, in a 5% CO_2 atmosphere. Total cell counts and viable cell number were determined using Trypan Blue Stain and a standard hemocytometer chamber.

Only freshly isolated cells were used for the cell culture experiments. Collagen coated carrier polymer samples were prepared by air drying a solution of collagen I ($32\mu g/cm^2$) in 0.1 M acetic acid (200μg/ml) in a laminar flow cabinet overnight.

Cells were seeded at densities of 12.0×10^6 cells/ml onto triplicates of the surfaces using a 10 mm diameter stainless steel ring to keep the samples flat in 6 well tissue culture plates. After 24h in culture, the keratinocyte attachment on one sample from each triplicate was determined using an MTT-ESTA assay ( ). This estimates the viable cell number, the assay having previously shown to parallel increases in cell number for human keratinocytes ( ) . Cells were incubated with 0.5 mg ml^{-1} of MTT in PBS for 40 min. The stain was then eluted with acidified isopropyl alcohol. An optical density measurement was then made at 540 nm with a protein reference wavelength of 630 nm which was subtracted.

The remaining two samples from each triplicate were placed in contact with DED and Green's media added so that the surfaces sat at the air/liquid interface. The DED/surface wound bed models were placed in an incubator at 37°C for 4 days, after which the surfaces were separated from the DED and the level of cell transfer from surface to DED assessed using the MTT assay, as described above. MTT of the DED required that the DED was incubated with MTT for 120 mins before elution of the stain.

Results

XPS characterisation

XP survey scan spectra of PCPs prepared from acrylic acid and 1,7-octadiene revealed only carbon and oxygen in the deposits. The O/C ratios are shown in Table 1. The O/C ratio increased as the mole fraction of acrylic acid in the monomer feed increased. The C 1s core level spectrum of the PCP was peak

fitted for various oxygen-containing functionalities. First, spectra were corrected for sample charging, setting the hydrocarbon signal to 285 eV. The following functionalities were then fitted: alcohol/ether (C-OH/R) at a shift of +1.5 eV; carbonyl (C=O) at +3.0 eV; carboxylic acid/ester (COOH/R) at +4.0 eV; and a β-shifted carbon bonded to carboxylate (C-COOH/R) at +0.7 eV. The results of peak fitting are shown in Table 1 and an example peak fit ($F_{as}/F_{tot} = 1$). In the peak fit the FWHM of component peaks were kept equal and were in the range 1.4-1.6 eV. The Gaussian to Lorentzian ratio (G/L) of the component peaks were also kept constant and were in the range 0.8-0.9. While XPS cannot distinguish between carboxylic acid and ester groups, grazing angle infra-red spectroscopy of plasma polymerised acrylic acid has shown, that at the low powers employed in this study, the carboxylate peak in the XP spectra can be assigned to carboxylic acid rather than ester ( ). Other carbon-oxygen functionalities present in the PCPs (besides carboxylic acid) include carbonyl and alcohol/ether. These arise as a result of fragmentation of the monomer in the plasma. Reaction between the deposit and water desorbed from the walls of the plasma vessel (during polymerisation) and atmospheric oxygen and water (after polymerisation) also contribute. The C-OH/R peak is thought to be predominantly hydroxyl. In a previous study we examined the identity of the oxygen-containing functionalities in PCPs of acrylic acid/1,7-octadiene (prepared with varying molar fractions of acrylic acid in the monomer feed) in more detail ( ) Based on this study, we believe that on the PCP surface, keratinocytes respond to the carboxylic acid functionality, and not C-OH. The latter has to be present in high concentrations (25%) to promote cell attachment ( ).

Table 1 Summary of XPS results for PCPs prepared from acrylic acid and 1,7 octadiene

		% functionality in C1 s core level			
F_{aa}/F_{tot}	O/C ratio	C-C, C-H	C-OR	C=O	COOH/R
0	0.04	95.8	4.7	-	-
0.25	0.11	88.4	5.7	1.0	2.6
0.5	0.16	87.1	1.4	1.2	5.4
1.0	0.51	50.4	6.4	1.0	21.1
carrier	0.47	52.2	1.2	16.0	16.0

A β-shifted carbon bonded to carboxylate ((C-COOH/R) at +0.7 eV from the hydrocarbon) of equal magnitude to the carboxylate has been added to the peak fit.

Cell attachment on surfaces

After isolation of the keratinocytes a cell count was performed using a hemocytometer which showed 97% cell viability (2.5×10^7 total cells). After 24 h the surfaces were examined using an MTT assay. The results are shown in Fig. 1. The data show that acid containing surfaces prepared with 50% and 100% acrylic acid in the monomer flow performed slightly better than Collagen I. The surface made with 25% acid in the flow was comparable to TCPS, whilst keratinocyte attachment on the hydrocarbon surface was poor.

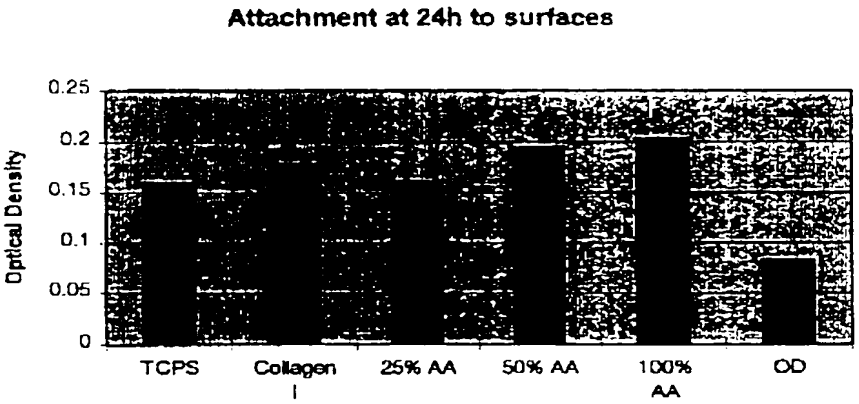

Figure 1.

Transfer of cells to DED

Table 2 summarises the results of separating the carrier polymer surfaces from the DED. The collagen I surface and the surface prepared with 100% in the gas flow were well adhered to the DED, indicating that substantial transfer of keratinocytes from the surface to the DED had occurred. Surfaces with lower amounts of acid in the monomer flow were less well adhered, whilst the carrier and hydrocarbon surfaces readily peeled apart from the DED, indicating lesser degrees of cell transfer had taken place.

Table 2 Adherence of surfaces to DED after 4 days in contact

Collagen I	Well adhered
Hydrocarbon	Peeled apart easily-no adherence
Carrier	Peeled apart easily-no adherence
25% Acrylic Acid	Adhered

50% Acrylic Acid	Adhered, but less than 25% surface
100% Acrylic Acid	Well adhered

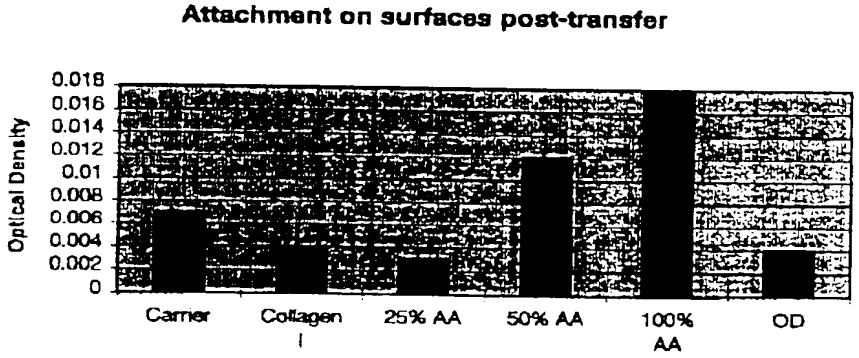

Figure 2

After 4 days of juxtaposition of the surfaces and the DED, the two were separated and Figure 2 shows the results of the MTT assay on the surface and Figure 3 of the MTT assay on the DED. The optical density of the cells remaining on the surfaces was extremely low in all cases compared to that seen for cells transferred to the DED. Cells grown on collagen 1 exhibited the highest value when examined for transfer to the DED, approximately 4 times greater than that seen for with carrier alone. For cells grown on the 25% acid treated surface transfer was comparable to that seen with cells grown on carrier alone. Cells grown on the 50% and 100% acid treated, surfaces showed however significantly greater transfer to the DED. Cells grown on the hydrocarbon treated surface showed very little transfer to the DED.

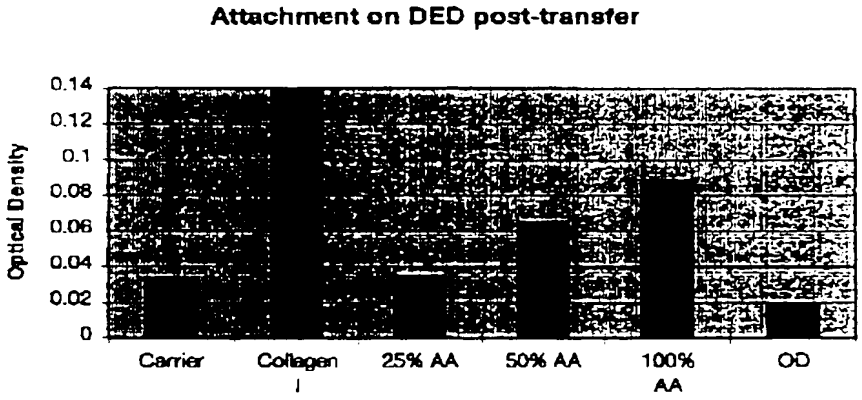

Figure 3

Discussion

The purpose of this study was to extend previous work from this laboratory, which had disclosed the use of PCP surfaces for keratinocyte attachment and proliferation, but not addressed the transfer of these cells to DED. Keratinocytes represent a particular challenge for such studies because they will undergo irreversible terminal differentiation on many substrates. Such cells lose the capacity to migrate or form colonies - properties which are required in considering transfer of keratinocytes from supporting surfaces to wound beds to achieve re-epithelialization. Accordingly, our aim was to examine to what extent surfaces which promoted attachment would encourage transfer of cells in a simple in vitro wound model.

Human keratinocytes were successfully cultured on PCP surfaces containing varying concentrations of carboxylic acid groups, with the number of cells attached being comparable to the performance of cells on collagen I, a preferred substrate for keratinocyte culture.

The use of a hydrocarbon plasma polymer as a negative controls is important because a previous study has raised doubts about the suitability of TCPS as a control ( ). These concerns have arisen because of the surface treatments given to TCPS during manufacture, which may render TCPS unstable to aqueous solutions depending on the level of oxidation at the surface. It is unclear whether different batches of TCPS receive precisely the same amount of surface oxidation, or if this surface oxidation is susceptible to ageing.

Although the dependence of cell attachment on functional group concentration is yet to be fully explored, keratinocytes have been previously shown to have enhanced attachment on surfaces with low 2-3% amounts amounts of acid functionality ( ). However, in this study attachment is also shown to be high on a surface containing 21% acid. It should be recalled that the acid PCPs also contain other O-C functional groups, predominantly C-OH. Even so, our previous studies have demonstrated that acid PCPs are comparable to collagen I in terms of degree of confluency and cell number (as determined by DNA assay).

In aqueous media, the acid PCPs can hydrate, as we will demonstrate elsewhere ( ). The stability of acid PCPs has been shown to be dependent on the concentration of acrylic acid in the monomer flow. High concentrations of acid (>60% of the total flow) result in less stable surfaces. This requirement led to the development of low concentration acid surfaces (<5%) as being labelled "ideal" for promoting attachment and subsequent proliferation. However, with regard to cell transfer from acid surfaces, different criteria are likely to apply. Whilst low concentrations of acid groups

impart stability to the surface, the keratinocytes may be sufficiently well attached that transfer is inhibited. This assertion is borne out in the results of the transfer experiments, where 25% acid flow in the monomer feed (2.6% carboxylic acid at the PCP surface) showed the lowest degree of transfer to DED. In contrast, with 100% acid flow in the monomer (>20% carboxylic acid at the PCP surface), transfer of cells was significantly higher. These surfaces were only outperformed by Collagen I. With 50% acid in the monomer flow, transfer was intermediate between the high and low acid functionality surfaces, as would be expected. These results indicate that the optimum surfaces for promoting attachment and proliferation may not be those which result in the largest degree of keratinocyte transfer from PCPs to DED. The low amount of transfer from the hydrocarbon PP confirm that such a surface is not capable of supporting keratinocytes in a proliferative state. Although the dependence of cell transfer on functional group concentration is yet to be fully explored, keratinocytes show enhanced transfer from surfaces with high amounts of acid functionality. It is clear therefore that there exists a compromise between surfaces which promote proliferation (low acid functionality), and those which promote transfer (high acid functionality).

In serum-containing medium, it has been shown that cells respond to an adsorbed layer of protein, rather than directly to the substratum itself ( ). This interfacial protein layer adsorbs (almost) spontaneously. The differences in cell response to the substrata under investigation suggest that there are either changes in the composition of the protein films that adsorb or in the activities of these proteins after adsorption, or a combination of both of these. Cell attachment has been shown to be supported by a number of adhesive proteins, such as fibronectin and vitronectin. Ratner et al ( ) have shown

differences in the protein layers that develop on SAMs with alkanethiolates of different terminal chemistries and that these in turn support different levels of bovine aortic endothelial cell attachment.

Whilst cell attachment and spreading are important conditions for cell proliferation, they are not exclusive conditions. Serum is also a source of growth factors and these have been shown to be essential for the proliferation of primary mammalian cells. It has been suggested that the adsorption of growth factors onto extracellular matrix material plays a role in their activation ( ).

Based upon the above discussion, it is evident that the success of the acid PCPs in supporting keratinocyte attachment and transfer is multi-factorial. However, our results would indicate that keratinocyte attachment and transfer are promoted specifically by the carboxylic acid functionality. This is most probably through control of the interfacial protein layer that forms from serum.

Conclusions

PCP surfaces containing high concentrations of acid groups (typically carboxylic) encouraged keratinocyte attachment and transfer to DED compared to hydrocarbon surfaces. The initial attachment of cells on surfaces containing ~20% acid groups was comparable to that of cells on collagen I substrates after 24h in culture.

Cell transfer from PCPs to DED was greatest for surfaces containing high concentrations of carboxylic acid functionality, although transfer was also observed from surfaces with low acid functionality concentration.